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Keksinnön nimitys
Title of invention

"Method for identifying an individual at risk for vascular and cancer disease"
(Menetelmä verisuoni- sekä syöpätaudille riskialttiin yksilön identifioimiseksi)

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METHOD FOR IDENTIFYING AN INDIVIDUAL AT RISK FOR VASCULAR AND CANCER DISEASE

Field of the invention

The present invention relates to a combination diagnostic method for identifying individuals who are at risk for coronary and vascular, as well as cancer diseases.

The method according to the invention is based on the combination of two tests carried out on a blood or serum sample in order to identify individuals prone to develop or exhibiting elevated levels of homocysteine, which individuals are thus at risk of developing afflictions resulting from such elevated levels, such as cardio- and cerebrovascular disease, including atherosclerosis and ischaemic stroke, as well as cancer diseases.

Background of the invention

A number of different gastric diseases or conditions, such as chronic atrophic gastritis, pernicious anaemia, ventricular ulcer, gastric polyposis and the Ménétrier disease (giant hypertrophic gastritis) precede gastric cancer. Clearly identifiable changes of the mucosa are dysplasia and adenoma. It has been established that in almost all diseases the risk is mediated over chronic atrophic gastritis.

Chronic gastritis means a prolonged inflammatory condition of the gastric mucosa. The disease can coarsely be divided into the superficial and the atrophic form. In superficial gastritis, the inflammatory cell infiltration is concentrated below the surface epithelium. In case the inflammation progresses and diffuses between the specific gastric secretory glands, one refers to chronic atrophic gastritis. In such a case, the normal glandular structures of the gastric mucosa are at least partly substituted by metaplastic changes.

The relative risk of gastric cancer in patients suffering from atrophic gastritis in the corpus area of the stomach has been estimated, as calculated from the Finnish cancer statistics, to be about 4- to 5-fold as compared to persons having a healthy mucosa. In addition, there is a risk for falling ill with pernicious anaemia due to intrinsic factor deficiency and B12 vitamin absorption disturbance. In severe atrophy of the antrum area, the risk is even 18-fold. If atrophic changes appear both in the antrum and the corpus area (pangastritis), the risk can increase to even 90-fold.

The publication WO 96/15456 discloses a method for screening for the risk of gastric cancer according to which atrophy in the mucosa either of the corpus or the antrum area, or of both, is determined by determining the pepsinogen I (PGI) and gastrin-17 (G-17) analyte levels in a serum sample, and comparing the levels so determined to a method-specific cut-off value for respective analyte. The levels determined are preferably also compared to a method-specific reference value for respective analyte.

A serum PGI value below the specific cut-off value for PGI indicates atrophic gastritis in the corpus area of the stomach. If the serum G-17 concentration is below its cut-off value, the atrophy is located in the antrum area of the stomach. In pangastritis, the serum PGI is below the cut-off value and the serum G-17 value is at the lower limit of its reference value.

Methylenetetrahydrofolate reductase (MTHFR) is an intracellular enzyme that is needed for remethylation of homocysteine to methionine. Impaired function of this enzyme is caused by defects in the structure of the MTHFR gene, or by nutritional deficiencies of *i.a.* folate, vitamin B6 and/or vitamin B12. Impaired function of the MTHFR enzyme results in an increase in the serum/plasma level of homocysteine (homocysteinemia) and in homocysteinuria. Increased serum/plasma levels of homocysteine has in many studies shown to be associated with an increased risk for various coronary and vascular diseases, a high se-

rum/plasma level of homocysteine above the reference value being a serious independent risk factor for coronary and vascular disease and ischaemic stroke.[1-5]

The atherogenic influence of homocysteine is thought to be based on an increased production of reactive oxygen species which enables lipid peroxidation.
The supply of sufficient B12 vitamin is necessary for folate metabolism and normal blood production, as well as for the function of the nerve cells. Vitamin B12 forms a complex with a protein, the intrinsic factor, produced by the mucosa of the corpus area of the stomach, which complex is resorbed in the lower part of the ileum. This is the preferred resorption form of vitamin B12.

A deficiency of intrinsic factor, as a result of atrophic gastritis or stomach cancer, especially in the corpus area of the stomach, will ultimately lead to a deficiency in B12 vitamin in the body and hence to an increase in the homocysteine concentration. It would thus be highly valuable to identify those subjects who have or are at a high risk for vitamin B12 deficiency due to atrophic gastritis, and who therefore might have an elevated serum or plasma level of homocysteine. In those persons an early initiation of vitamin B12 supplementation would be beneficial in the prevention of vascular diseases. It would also be valuable to be able to identify those individuals which would be at risk of cancer due to overproduction of intracellular oxygen radicals, and in whom a B12 vitamin supplementation or other treatment could be beneficial.

Summary of the invention

The present invention is directed to a method for combining an assay of determining, in a serum sample, a marker for atrophic gastritis in combination with an assay for homocysteine, in order to assist in the diagnosis of, or in the determination of the risk of vascular, as well as cancer diseases in an individual, the term vascular being understood broadly to include any coronary or vascular disease which can result from the atherogenic influence of homocysteine.

The method according to the invention is a method for identifying an individual at risk for vascular and cancer disease, the method comprising the steps of

- determining quantitatively the pepsinogen I (PGI) analyte concentrations in a serum sample from said individual,
- determining a method specific cut-off value for the said analyte,
- comparing the analyte concentration so determined to the method-specific cut-off value for the analyte, and
- determining the homocysteine concentration in a serum sample from the individual, and comparing it to a method-specific reference value for homocysteine.

The determination of the serum PGI and homocysteine concentrations can take place in any order, in order to simultaneously obtain knowledge of both the serum PGI and homocysteine levels for the purpose of assisting in the diagnosis of, or assessing the risk of vascular disease or cancer. However, according to an embodiment of the invention, the serum PGI concentration is determined and compared to its determined or chosen method-specific cut-off value, and for further diagnosis an individual is selected who has a serum PGI concentration value below its method-specific cut-off value. In this embodiment, only the individuals which exhibit low serum PGI values indicative of corpus atrophy, are screened for homocysteine.

According to one embodiment of the invention, also the B12 concentration is determined in the serum of the said individual, and compared to a method-specific reference value for vitamin B12.

Thus according to a preferred embodiment, the present invention is especially aimed at screening such individuals, which still do not exhibit a B12 vitamin deficiency, that is which exhibit substantially normal B12 vitamin levels, but which due to low PGI values are diagnosed for atrophy in the corpus area of the stomach and which also have high homocysteine serum levels. Such an identification makes it possible to already at an early stage resort to preventative measures,

for example to B12 supplementation, in order to counteract the development of the afflictions associated with elevated homocysteine levels.

Detailed description of the invention

1. Determination of pepsinogen I (PGI)

The method of determining PGI in a serum sample can be carried out as is described in the publication WO 96/15456, which publication is included herein for reference.

The said method preferably includes the steps of using poly- or monoclonal antibodies to pepsinogen I in an immunological method for the determination of pepsinogen I. Suitably the reaction is carried out on a suitable support, such as a plastic, glass or cellulose support, for example on a microplate. The immunological methods can be carried out in a known manner, using e.g. absorbance, luminescence or fluoresence techniques for measuring the said pepsinogen I concentration in the sample.

If the serum pepsinogen I concentration is below the cut-off value, which depending on the specificity and sensitivity agreed upon for the method in question, is 20-30 μg/l, which corresponds to appr. 450 - 690 pmol/l, there is atrophy in the corpus area of the stomach. The normal or reference value for PGI is in the range of 25 - 120 μg/l.

2. Determination of homocysteine

Homocysteine levels in serum can be determined according to any of the methods known *per se* for this purpose and which are also commercially available, *e.g.* in kit form. The established method for quantifying total homocysteine in plasma or serum is high performance liquid chromatography with radioactive, fluorescent or electrochemical detection. Also an enzyme immunoassay (EIA) method has been

developed (Bio-Rad Laboratories; Axis-Shield A/S) as well as a fluorescence polarisation immunoassay (FPIA; Abbott Laboratories), the immunoassay including pretreating the specimens with dithiothreitol and adenosine, followed by an enzymatic step to form S-adenosyl-L-homocysteine, and total homocysteine is measured e.g. using monoclonal anti-S-adenosyl-L-homocysteine antibodies, see *e.g.* US 5,631,127.

The reference values for homocysteine is to some degree method-specific, but generally varies between appr. 5 to 15 μmol/L. A serum homocysteine level which is above the method-specific reference level for homocysteine is taken as constituting a risk factor, as explained above. A homocysteine level of above 15 μmol/L can in most cases be considered to constitute such an elevated level constituting a clear risk factor.

3. Determination of B12-vitamin

According to the invention, the method of diagnosis includes an optional method of determining the B12 vitamin concentration in the serum sample. The B12-vitamin (cobalamin) concentration can be determined according to any of the methods *per se* known for this purpose. Such known methods include microbiological assay of serum B12 employing an organism, such as *Euglena gracilis* or *Lactobacillus leichmannii* which requires cobalamin for growth. Also radioisotope dilution assays for B12 vitamin have been utilized and such assay techniques are well documented in the literature, e.g. Lau *et al.*, "Measurement of serum B12 levels using Radioisotope Dilution and Coated Charcoal", Blood, 26 (1965), 202. Radioisotope dilution methods are more rapid and give results comparable with those of e.g. the *Euglena* assay, provided the binding protein is specific for biologically active cobalamin. A standardized pure or purified intrinsic factor preparation is most satisfactory as the binding protein as it binds specifically to true cobalamin rather than cobalamin analogues.

The radioisotope dilution assay of B12 generally includes the step of freeing endogenous B12 from its natural binding protein e.g by boiling at a selected pH and then adding a measured amount of the radioisotope 57Co-B12, and a limited amount of binding protein. All of the binding protein will be bound by some form of B12 since the amount of radioisotope B12 added is sufficient to bind the small amount of protein. As both the natural and the radioactive B12 compete to bind with the binding protein, the degree to which the radioactive count of the protein bound B12 was inhibited is indicative of the amount of B12 in the sample. This method has been modified by Lau, *supra,* by separating unbound B12 from protein bound B12 by protein coated charcoal and the radioactivity of the supernatant liquid containing the mixture of bound radioactive B12 and bound non-radioactive B12 is counted for radioactivity. The serum B12 concentration is then calculated from the count, often by comparison with a standard chart. Radioassay kits are commercially available for carrying out the method.

B12 vitamin deficiency has also been determined using e.g. chemiluminiscence receptor assays (Wentworth, S. *et al.,* Clin. Chem., vol 40 537-540), radioimmunoassays (Endres, D.B., *et. al.* Clin. Chem., Vol. 24, 460-465) as well as nonisotopic binding assays, CEDIA, cloned enzyme donor immunoassays (van der Weide, J. *et al.* Clin. Chem., Vol. 38, 766-768).

The reference value for B12 varies between 200-900 ng/l, corresponding to appr. 170 to 700 pmol/l.

In our recent unpublished work we found that 50% of those subjects suffering from corpus atrophy (SPGI < 25 μg/l) whose vitamin B12 serum concentration was below the lower part of the reference limit ($<$ 170 pmol/l) had markedly increased serum homocysteine concentration (mean 33.3 μmol/l, 16-157 μmol/l). In addition, 22% of those subjects whose vitamin B12 serum concentration was between 180 - 230 pmol/l (reference values 170 - 700 pmol/l) had also an increased homocysteine concentration (mean 18.9 μmol/l, 16 - 25 μmol/l).

The present invention also relates to a kit for use in the method according to the invention the kit comprising

- means for determining the PGI concentration in a serum sample,
- means for determining the concentration of homocysteine in a serum sample.

The kit according to the invention can comprise a combination of the individual components needed to quantitatively determine the serum pepsinogen I and the homocysteine concentration in a blood serum sample. For this purpose the kit can comprise separate vials or containers for the necessary components, such as antibodies and substrates to be used for the determination of the analyte.

References

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Claims

1. Method for identifying an individual at risk for vascular and cancer disease, the method comprising the steps of

- determining quantitatively the pepsinogen I (PGI) analyte concentrations in a serum sample from said individual,
- determining a method specific cut-off value for the said analyte,
- comparing the analyte concentration so determined to the method-specific cut-off value for the analyte, and
- determining the homocysteine concentration in a serum sample from the individual, and comparing it to a method-specific reference value for homocysteine.

2. The method according to claim 1, comprising determining the serum PGI concentration, and selecting for further determination of the homocysteine concentration an individual who exhibits a serum PGI concentration below its cut-off value.

3. The method according to claim 1 or 2, comprising the further step of determining the B12 vitamin concentration in the sample and comparing it to a method-specific reference value.

4. Kit for carrying out the method according to claim 1, comprising

- means for determining the PGI concentration in a serum sample,
- means for determining the homocysteine concentration in a serum sample.

5. The kit according to claim 4 wherein the means for determining the pepsinogen I and homocysteine concentration comprise immunological means.

(57) Abstract

The present invention is aimed at a method and a kit for identifying an individual at risk for vascular disease and cancer, the method comprising the steps of determining quantitatively the pepsinogen I (PGI) analyte concentrations in a serum sample from said individual, determining a method specific cut-off value for the said analyte, comparing the analyte concentration so determined to the - method-specific cut-off value for the analyte, and determining the homocysteine concentration, in a serum sample from the individual.